

Effect of Acetone Fixation on Infectivity and Antigenicity of Respiratory Syncytial Virus and Adenovirus in the Fluorescent Antibody Test

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An investigation was made on the effect of acetone fixation on infectivity and immunofluorescent antigenicity of respiratory syncytial virus and adenovirus type 5, lipid- and nonlipid-containing viruses, respectively. Viruses were allowed to replicate in HEp-2 cells, and the cells were then fixed in acetone at 5 C for periods ranging from 30 s to 7 days. Treatment for 10 min was sufficient to inactivate respiratory syncytial virus, whereas infectious adenovirus type 5 could be isolated from cells immersed in acetone for 7 days. There was a gradual reduction, to 50% of that observed at 30 s, in the intensity of fluorescent antibody staining of both viruses with increasing fixation time, but no significant decreases in fluorescent antibody end-point titers of antisera to either virus were observed.

In the fluorescent antibody technique using intracellular virus as the antigen, the fixative should give good preservation of host cells and also inactivate the infectivity of the virus without destroying its antigenicity (6). Common histological fixatives such as ethanol and formalin are generally not used for viral immunofluorescent procedures and have been reported to alter the antigenic activity of many of the viruses that have been studied (3, 5, 9). Although acetone is the most frequently used fixative for viruses (3, 6, 8), relatively little information is available concerning infectivity and antigenic stability of viruses fixed in acetone. Kundin and Liu (5) found that placing infected sections in acetone inactivated 99% of West Nile virus within 8 min; but antigenicity, determined by intensity of fluorescent staining, remained undiminished with fixation for periods up to 4 days. Spendlove et al. (9) reported that there was no loss in intensity of fluorescent staining of reovirus-containing cells fixed in acetone for 5 min, but did not indicate if infectious virus was present or not. In contrast to these observations, Metzger et al. (7) found that the antigenicity of Venezuelan equine encephalomyelitis virus in tissue culture was unstable after fixation for 15 min in acetone, and Banatvala et al. (1) used isopentane as a fixative after obtaining unsatisfactory results with acetone-fixed preparations for titrating immunoglobulin M antibody to Epstein-Barr virus.

The purpose of this study was to ascertain if acetone would destroy the infectivity of respiratory syncytial virus and adenovirus type 5, lipid-containing and nonlipid-containing viruses, respectively, without having an adverse effect on the intensity of staining or titration end point in the indirect immunofluorescent test for antibodies to the viruses.

MATERIALS AND METHODS

Cell cultures. HEp-2 cells were grown at 37 C on Eagle minimal essential medium supplemented with 10% fetal calf serum, and maintained on Eagle minimal essential medium with 1% fetal calf serum. All media contained 100 U of penicillin and 100 µg of streptomycin per ml. Subcultures were prepared by treating cell monolayers with 0.02% versene and then resuspending dispersed cells in growth medium.

Viruses. Preparations of respiratory syncytial virus (Long strain) and adenovirus type 5 (adenoid 75 strain) were obtained from the American Type Culture Collection and propagated by serial passage in HEp-2 cells. Respiratory syncytial (RS) virus was harvested from the culture fluid of infected monolayers showing maximum cytopathic effect (CPE). After replication adenovirus 5 does not have an immediate cytolytic effect, and after the appearance of advanced CPE the amount of extracellular virus is small compared to the quantity of intracellular virus (2, 4). Therefore, progeny virions of adenovirus 5 were collected from cells disrupted by freezing and thawing three times.

Antigens and antisera. Monolayer cultures of HEp-2 cells were inoculated with either RS virus or adenovirus 5. After a 4-h absorption period at 37 C,

the inocula were removed and the cells were reincubated with maintenance medium. When the cells showed distinct CPE they were harvested by treatment with versene, washed three times, and resuspended in phosphate-buffered saline (PBS), pH 7.2. Cells were then placed on microscope slides, air-dried for 15 min at 37 C, fixed in acetone at 5 C for various periods ranging from 30 s to 7 days, and again air-dried.

Acute and convalescent sera from persons with known RS virus or adenovirus infection were obtained from the Virology Section, Wisconsin State Laboratory of Hygiene, Madison, Wisc.

Isolation of infectious virus from acetone-fixed cells. Uninfected and virus-infected cells that had been fixed and air-dried were washed with PBS, scraped from the slides, and inoculated into tubes containing monolayers of HEP-2 cells. Approximately 2.5×10^6 fixed cells in 1.0 ml of maintenance medium were used for inoculation. Fixed cellular material was allowed to remain in contact with the HEP-2 cells for 18 h at 37 C, after which the material was decanted and the cultures were reincubated with maintenance medium. The cultures were then observed for the appearance of typical RS virus and adenovirus CPE. Cells exhibiting CPE were assayed for infectious virus. Cultures that were negative for CPE were maintained for a 40-day period after inoculation by subculturing at 10-day intervals. The subcultured cells were placed on growth medium for 3 days and then changed to maintenance medium until the next subculture.

Indirect immunofluorescent technique. Twofold dilutions, starting at 1:2, of human antisera to either RS virus or adenovirus 5 were prepared in PBS and layered on uninfected and appropriate antigen-containing cells. The cells were incubated in a humid atmosphere at 37 C for 1 h. The slides were then washed in three changes of PBS over a 15-min period, rinsed in distilled water, and air-dried. Fluorescein-labeled goat anti-human immunoglobulin G was then added to the cells. After incubation for 1 h at 37 C, the slides were washed in PBS and counterstained in 0.5% Evans blue for 30 min. The slides were again washed in PBS, rinsed in distilled water, air-dried, and mounted in buffered glycerol, pH 7.2. The fluorescein-anti-globulin conjugate was purchased from Kallestad Laboratories, Chaska, Minn. It contained 10 mg of protein per ml, had a fluorescein-protein ratio of 2.3, and was used at a predetermined optimum dilution of 1:10 in PBS.

Preparations were examined for fluorescence with a $\times 100$ oil objective on a Leitz Wetzlar microscope equipped with an Osram HBO 200-W mercury vapor lamp, an immersion dark-field condenser, a UG 1 exciter filter, and a matched ultraviolet-absorbing barrier filter.

RESULTS

Isolation of infectious virus from fixed cells. Infectious RS virus was recovered from cells fixed in acetone at 5 C for 30 s, but could not be isolated from preparations immersed 10

min or longer in the fixative. Infectious adenovirus 5 was present in cells fixed for all periods tested ranging from 30 s to 7 days (Table 1). Positive and negative controls consisting of virus-infected cells that were not fixed and uninfected cells that were treated with acetone gave fitting results. Although adenovirus 5 could be isolated from host cells even after 1 week in acetone, there was a decrease in virus infectivity titer with increasing fixation time. Adenovirus 5 infectivity titers were unchanged for fixation periods of 30 s to 1 h, but showed a gradual decline thereafter (Table 2). There were no changes in the titer of adenovirus 5 due to incubation for up to 1 week at 5 C. A suspension of adenovirus 5 in cell culture medium was divided into two portions. One portion designated 0 h was immediately frozen at -70 C, and the other was incubated at 5 C for 7 days. Simultaneous infectivity titrations were made on both portions, and were $10^{3.4}$ and $10^{3.6}$ mean

TABLE 1. Isolation of infectious RS virus and adenovirus type 5 from HEP-2 cells fixed in acetone at 5 C for various periods

Fixation time	RS virus	Adenovirus 5
30 s	+	+
10 min	-	+
1 h	-	+
3 h	-	+
6 h	-	+
1 day	-	+
2 day	-	+
3 day	-	+
4 day	-	+
5 day	-	+
6 day	-	+
7 day	-	+

TABLE 2. Decrease in titer of infectious adenovirus type 5 with increasing fixation time in acetone at 5 C

Virus fixation period	Virus titer (TCID ₅₀ /0.1 ml) ^a
30 s	$10^{5.6}$
10 min	$10^{5.8}$
1 h	$10^{5.5}$
6 h	$10^{5.0}$
1 day	$10^{3.2}$
3 day	$10^{3.0}$
7 day	$10^{2.1}$

^a The mean tissue culture infective dose (TCID₅₀) was calculated 10 days after inoculation of tubes containing HEP-2 cells with 0.1 ml of serial 10-fold dilutions of virus. Four tubes were inoculated for each dilution. A virus preparation that had not been treated with acetone was assayed for infectious virus at 0 h and after 7 days at 5 C. There was no change in adenovirus titer due to incubation at 5 C for 7 days.

tissue culture infective dose U per 0.1 ml for 0-h and 7-day portions, respectively.

Effect of acetone on immunofluorescent antigenicity of viruses. To examine intensity of fluorescence, the weakest dilutions of acute and convalescent antisera giving maximum staining were applied to virus-containing cells that had been exposed to acetone for various times. Although RS virus infectivity was destroyed within 10 min of placing preparations in acetone, the intensity of staining in the indirect immunofluorescent test for antibody to the virus remained undiminished with fixation of the antigen for as long as 24 h. The amount of fluorescence with RS virus fixed for 7 days was about 50% of that observed with antigen fixation for 30 s (Table 3). There were no changes in the intensity of fluorescence with adenovirus 5 preparations fixed for up to 72 h, after which there was a gradual reduction in brilliance of staining resulting in no more than a 25 to 50% loss with antigen-containing cells removed after 7 days in acetone (Table 3).

The data presented in Table 4 demonstrate that there were no significant differences between the fluorescent antibody end-point titers obtained with viruses fixed for short or long periods.

Nonspecific fluorescence was not a problem in the above studies. Counter-staining with 0.5% Evans blue for 30 min was as effective as

TABLE 4. *Fluorescent antibody end-point titrations of antisera to RS virus and adenovirus type 5 using viral antigens fixed in acetone at 5 C for various periods^a*

Antigen fixation time	Paired sera from patient with RS virus infection		Paired sera from patient with adenovirus infection	
	Acute	Convalescent	Acute	Convalescent
30 s	16	512	32	256
10 min	16	256	32	256
1 h	16	512	32	256
3 h	16	512	32	256
6 h	16	512	32	256
1 day	16	512	32	256
2 day	16	512	32	256
3 day	16	256	32	256
4 day	8	256	16	256
5 day	8	256	16	128
6 day	8	256	16	128
7 day	8	256	16	128

^a Titers are given as reciprocals of serum dilutions.

absorption of antisera with uninfected cells for elimination of nonspecific staining.

DISCUSSION

The present investigation demonstrates that acetone destroys the infectivity of RS virus in less than 10 min. In contrast, although there was some loss in titer, infectious adenovirus 5 could be isolated from host cells immersed in the fixative at 5 C for 7 days, the longest period tested. These results are not unusual since acetone is a lipid solvent, and RS virus contains essential lipids, whereas adenoviruses do not. In addition to being lipid-free, adenovirus 5 is stable and showed no decrease in infectivity titer due to incubation at 5 C for 1 week.

The presence of infectious adenovirus 5 in fixed cells draws attention to the need for care when using such preparations in the fluorescent antibody test. Besides members of the human adenovirus group, some of which are oncogenic for certain animals (10), other nonlipid-containing viruses might be resistant to inactivation of their infectivity by acetone.

Acetone had little detrimental effect on the antigenic determinants of either RS virus or adenovirus 5 in the indirect immunofluorescent procedure for detection and quantitation of antibodies to the agents. No changes occurred until after fixation for much longer periods than the 5 to 30 min normally used for processing viral antigens. Fluorescent antibody titers of acute and convalescent sera to RS virus and adenovirus 5 were consistently one dilution less

TABLE 3. *Intensity of fluorescent antibody staining of RS virus and adenovirus type 5 fixed in acetone at 5 C for various periods^a*

Antigen fixation time	Paired sera from patient with RS virus infection		Paired sera from patient with adenovirus infection	
	Acute	Convalescent	Acute	Convalescent
30 s	4+ ^b	4+	4+	4+
10 min	4+	4+	4+	4+
1 h	4+	4+	4+	4+
3 h	4+	4+	4+	4+
6 h	4+	4+	4+	4+
1 day	4+	4+	4+	4+
2 day	3+	3+	4+	4+
3 day	3+	3+	4+	4+
4 day	3+	2+	3+	3+
5 day	2+	2+	3+	2-3+
6 day	2+	2+	2+	2-3+
7 day	2+	2+	2+	2-3+

^a Acute and convalescent antisera were used at predetermined optimal dilutions of 1:2 and 1:8, respectively.

^b 4+ = maximum intensity.

with antigens fixed for several days as compared to titers obtained with antigens fixed for less than 24 h. In addition, there was some reduction in brightness of fluorescent antibody staining when using antigens treated with acetone for prolonged periods. It is noteworthy that antibody end-point titration to determine the weakest serum dilution giving fluorescence was found to be a more preferable method than estimation of intensity of staining (5, 7) when studying the effect of acetone on virus antigenicity.

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